REMARKS

Claim Amendments

Applicants cancelled all of the previously presented claims and added new Claims 32-85. The new claims correspond directly to the elected Group I of original Claims 1-25 and 29-31 and do not go beyond the elected group. Specific support for the new claims in the original application as filed is indicated in the table below.

New Claim	Support in Specification/Original Claims
32	original Claim 1, with clerical amendment to clarify that the protein has at least one free cysteine - supported on page 7, line 35 to page 8, line 7
33	original Claim 2
34	original Claim 3
35	original Claim 1 and page 11
36	original Claim 2
37	original Claim 3
38	original Claim 1 and page 10, lines 32-38 and page 11, lines 15-17
39	original Claim 3
40	original Claim 7 and Claim 4
41	original Claim 3
42	original Claim 4
43	original Claim 5
44	original Claim 6
45	original Claim 4
46	original Claim 7
47	original Claim 8
48	original Claims 9, 10 and 11
49	original Claim 13 and page 9, line 24
50	original Claim 14
51	original Claim 15
52	original Claim 16
53	original Claim 17
54-55	page 11, lines 15-17
56	original Claim 18 and Claim 29

57	original Claim 30
58	original Claim 31
59-61	page 7, line 38 to page 8, line 3
62	original Claim 20; page 14, lines 25-29; new product by process limitation
63	original Claim 22 and page 13, lines 12-15
64-65	page 36, Example 14
66	original Claim 21 and page 13, lines 10-12
67-68	page 36, Example 14
69	original Claim 22, new product by process limitation
70	original Claim 23 and page 13, lines 17-19
71-72	page 49, Example 18D
73	original Claim 23 and page 13, lines 13-17
74-75	page 49, Example 18D
76	original Claim 24 and page 14, lines 24-27
77	page 13, lines 29-35
78	page 13, Examples 21-22
79	original Claim 25 and page 13, lines 23-26
80-81	page 67, Example 22
82	page 63, lines 4-6 and page 67, Example 22
83-84	page 67, Example 22, lines 20-23
85	original Claim 25 and page 13, lines 27-28

Restriction Requirement

The Examiner has considered Applicants' arguments in traverse of the Restriction Requirement, but has not been persuaded by the arguments and has therefore made the requirement final. Non-elected Claims 26-28 have been cancelled without prejudice to or disclaimer of the subject matter therein, and Applicants expressly reserve the right to pursue the subject matter of the non-elected claims in divisional applications without the need to file a terminal disclaimer.

Objection to the Specification and Rejection of Claims 8-14 and 20-25 Under 35 U.S.C. § 112, First Paragraph:

The Examiner has objected to the specification and rejected Claims 8-14 and 20-25 under 35 U.S.C. § 112, first paragraph, on the basis of written description. Specifically, the Examiner objects to the phrase "derivative or antagonist thereof" in the claims, contending that the instant specification does not describe the structure of such derivatives or antagonists.

Applicants traverse the rejection of Claims 8-14 and 20-25 under 35 U.S.C. § 112, first paragraph. Initially, in order to clarify the claims and to expedite prosecution, Applicants have removed the phrase found objectionable by the Examiner. Applicants submit, however, that this amendment is made solely to address the Examiner's concerns over what exact structures are inferred by the terms and to thereby clarify the dependent claims. The amendment is not intended to imply or admit that the method of Claims 32-41 is in any way limited to the specific protein that can be obtained or to exclude the production of antagonists or derivatives of known proteins. To the contrary, the method of the present invention can be used to obtain any soluble protein, wherein the protein has at least one added free cysteine.

In view of the foregoing remarks, Applicants respectfully request that the Examiner withdraw the rejection of Claims 8-14 and 20-25 under 35 U.S.C. § 112, first paragraph.

Objection to the Specification and Rejection of Claims 18-19 Under 35 U.S.C. § 112, First Paragraph:

The Examiner has objected to the specification and rejected Claims 18-19 under 35 U.S.C. § 112, first paragraph, on the basis of enablement. Specifically, the Examiner contends that Claims 18-19 are not enabled, because the protein produced by Claim 1 would result in a protein that is blocked at all free cysteines, such that one would not be able to attach a cysteine reactive moiety to the protein according to Claims 18 and 19.

To clarify the claims, the new set of claims incorporates the more specific method steps of Claims 29-31 to describe the modification of the cysteine with a cysteine reactive moiety. In these claims, a step of reducing the protein before exposing the protein to the cysteine reactive moiety is believed to obviate the Examiner's concerns.

In view of the foregoing remarks, Applicants respectfully request that the Examiner withdraw the rejection of Claims 18-19 under 35 U.S.C. § 112, first paragraph.

Rejection of Claims 1-25 and 29-31 Under 35 U.S.C. § 103:

The Examiner has rejected Claims 1-25 and 29-31 under 35 U.S.C. § 103, contending that these claims are unpatentable over Braxton (U.S. Patent No. 5,766,897) or Cox et al. (WO 95/32003) in view of Seeley et al. (EP 0 312 358). Specifically, the Examiner contends that Braxton discloses a method and composition for producing soluble proteins that have a free cysteine residue and a method of attaching PEG to the proteins. The Examiner also contends that Cox et al. teach IGF muteins having a free cysteine and PEG attached through the free cysteine. The Examiner admits that neither of Braxton or Cox et al. teach a method of producing a soluble protein having a free cysteine by exposing a host cell to the cysteine blocking agent so that the cysteine blocking agent forms a mixed disulfide with at least one cysteine residue and isolating the protein from the host. The Examiner cites Seeley as allegedly teaching a method of producing high yield bioactive monomeric protein by exposing a host cell to an oxidizing agent and then isolating the protein. Therefore, the Examiner asserts that it would have been obvious to combine the teachings of Braxton or Cox et al. with the teachings of Seeley et al. to define the claimed method. With regard to Claims 20-24, the Examiner contends that it would have been obvious to modify the site of PEG attachment and further, that it would have been expected to produce proteins having an improved EC_{50} because Cox et al. teach pegylated IGF muteins with improved EC_{50}.

Applicants traverse the rejection of Claims 1-25 and 29-31 under 35 U.S.C. § 103. As the Examiner admits, neither Braxton or Cox et al. teach exposing the proteins to a cysteine blocking agent, which forms a mixed-disulfide with at least one cysteine residue in the soluble protein. It is submitted that the differences between the methods of Braxton or Cox et al. and the method of the present invention are significant and that combination of either reference with Seeley et al. does not make up for the deficiencies of either reference. Specifically, the combination of references fails to teach or suggest a method wherein the proteins that are expressed by the host cell are soluble (i.e., proteins that are soluble in the absence of a denaturant), and wherein the step of exposing the soluble protein to the cysteine blocking agent (e.g., oxidizing agent) occurs prior to isolating the protein from

the host cell. Morever, in contrast to the methods of the cited references, the present method does not include a step of denaturing the protein. The cited references also do not provide the requisite motivation or expectation of success to arrive at the claimed invention, because each of the cited references describe methods that are suitable for producing insoluble proteins and do not appreciate the particular issues associated with production of soluble proteins or the advantages provided by the claimed invention.

First, with regard to Braxton et al. Applicants submit that the only chemical modification of a cysteine explicitly suggested by Braxton is to expose the cysteine variants, *after* they are isolated from the cell that produced them, to a maleimide-PEG reagent (column 37, lines 1-8). Maleimide-PEG is not a cysteine blocking agent according to the present invention because it forms a *permanent, non-reversible covalent bond* with a cysteine residue in a protein. This is not a useful reagent for producing a soluble protein with a free cysteine. The ability to reverse the cysteine blocking reaction (i.e., remove the cysteine blocking agent by partial reduction of the protein as set forth in Claim 59, for example), is an important step in the ability to then further manipulate the free cysteine, for example, by PEGylating the protein at the free cysteine residues following partial reduction of the protein.

In addition to not teaching the use of a cysteine blocking agent according to the present invention, Braxton does not teach the use of a cysteine blocking agent to modify cysteines in the protein at a step prior to the actual isolation of the protein, because they produce the insoluble protein before any chemical modification is applied. As discussed below, this is an important step in the method to produce soluble proteins having a free cysteine of the present invention, a step which is not appreciated or contemplated by Braxton.

Second, with regard to Cox et al., Applicants submit that the procedure taught by Cox et al. is also fundamentally different from the method of the present invention. Cox et al. discloses a method for refolding insoluble, inactive proteins into a soluble, active form by treating the insoluble proteins with a combination of a denaturing agent and a reducing agent, which steps are not taught, are not necessary and indeed, are not desirable, in the presently claimed method. More specifically, Cox et al. teaches the use of thiol-reactive compounds to *refold* an *insoluble, inactive* protein expressed in *E. coli*. The methods taught by Cox et al. are suitable for proteins that are insoluble and

inactive when expressed by a host cell, but are not useful for proteins that are expressed in a soluble form by a host cell. Cox et al., because they are working with an insoluble protein, teaches that the host cells expressing the protein should be lysed and insoluble proteins collected by centrifugation, *prior to any modification of the cysteine residues in the protein.* The reference further teaches that the supernatant containing the soluble proteins should be discarded. Since the proteins described in the present claims are expressed by the host cell in a soluble form, the proteins are present in the supernatant fraction of a cell lysate and would be discarded if one followed the procedure taught by Cox et al. Another difference from the presently claimed method is that Cox et al. teaches that the insoluble proteins should be denatured (with guanidine) and reduced (with DTT) to reduce disulfide bonds in the insoluble protein, and then treated with a combination of an oxidizing cysteine blocking agent (oxidized glutathione) and a reducing cysteine blocking agent (cysteine). The insoluble protein is then refolded to produce a soluble protein. This is fundamentally different than the method of the present invention, which does not recite, and indeed would not desire to use, a denaturing agent, or combinations of a reducing agent and an oxidizing agent, since these steps are particular to the production of the insoluble proteins. As discussed above with regard to Braxton, since the proteins described in the present application are soluble, they do not need to be denatured or refolded prior to exposure to a cysteine blocking agent, and such steps prior to addition of the cysteine blocking agent in the present method (i.e., before the protein was isolated) would be detrimental to the procedure and completely unnecessary.

Third, Seeley et al., even when combined with either of Braxton or Cox et al., does not make up for the deficiencies of these references. Seeley et al. teaches methods for promoting disulfide bond formation in isolated, insoluble, reduced, recombinant proteins that have been denatured to make them soluble. The recombinant proteins of Seeley et al. are inclusion bodies, which Seeley identifies as "insoluble, bio-inactive proteins" (column 1, line 21). Seeley does not teach or suggest that this method is useful for whole cells, soluble proteins (proteins that are soluble in the absence of a denaturant) or for proteins that have not been denatured first. According to the abstract and the Examples of Seeley et al., the recombinant proteins are *first* isolated as inclusion bodies from the host cell, then they are treated with a denaturing solution to denature the proteins. These steps occur *prior to* exposing the denatured protein to the oxidizing agent. Specifically, in column 5, line 33,

Seeley states that inclusion bodies are used in their process. The inclusion bodies are processed to remove contaminating proteins, lipids, and host cell debris to obtain relatively pure inclusion bodies. The purified inclusion bodies are then solubilized in a denaturant prior to treatment with the oxidizing agent. In column 1, lines 25-30, they state that inclusion bodies are recovered from the microbial cell and then dissolved in a denaturant.

In contrast to the methods described in the cited references, the first step of the claimed method requires that the proteins that are expressed by the host cell are soluble (i.e.,proteins that are soluble in the absence of a denaturant). Also in contrast to above-described methods, the present method includes a step of exposing the soluble protein to the oxidizing agent prior to isolating the protein from the host cell. This is the opposite of what is taught by the cited references. Furthermore, the present method does not include and would not include a step of denaturing the protein prior to exposure to a cysteine blocking agent. The soluble proteins obtained by the present invention do not require reduction or denaturation prior to treatment with the oxidizing agent. Indeed, using the present method, one can expect that most native disulfides are properly formed and only the free cysteine residue is not disulfide bonded. It is not a desirable feature of the claimed method to expose all of the cysteine residues in the protein to the cysteine blocking agent and it is not required, in contrast to the above-described methods for production of insoluble proteins.

Moreover, it is submitted that none of the references provides any motivation to make the combination as the Examiner has done, nor the requisite expectation of success at making and using the present invention. The present invention is explicitly directed to proteins made by a host cell in a soluble, active form, rather than to proteins that are made by a host cell in an insoluble, inactive form, as is primarily disclosed by the three cited references. As discussed above, the present method allows the reliable production of soluble proteins having free cysteines by incorporating method steps that are not taught by the cited references. Each of the cited references teaches that the host cells expressing the insoluble proteins should be lysed and insoluble proteins collected by centrifugation; the supernatant containing the soluble proteins should be discarded. The insoluble proteins are then denatured and reduced. The denatured and reduced proteins are then refolded and purified. None of these references provide any motivation to modify the method to address the production of soluble proteins or to expose the cells to a cysteine blocking agent prior to isolating

the protein from the host cell. Since the proteins described in the present application are soluble, they do not need to be denatured or refolded prior to exposure to a cysteine blocking agent, and it is undesirable in the present method to denature the proteins prior to addition of the cysteine blocking agent.

Furthermore, Seeley et al. actually provides a teaching away from combination with either of Braxton or Cox et al. to arrive at the invention, because Seeley suggests at column 4, lines 35-40, that it might be useful to replace cysteine residues with non-cysteine amino acids to avoid incorrect disulfide bond formation. This of course is a direct teaching away from the present invention, which is directed to the production of proteins with at least one added free cysteine.

Moreover, with regard to expectation of success at making and using the claimed invention, since the cited references do not teach the claimed method steps, none of the references, alone or in combination, can provide the skilled artisan with an expectation of success at using the claimed method. Cox et al. and Seeley et al. are not directed to the production of soluble proteins and teach no particular methods by which such proteins could be produced. Although the methods of Braxton could be extended generally to producing soluble proteins, Braxton does not actually teach method steps by which soluble proteins having a free cysteine can be reliably produced, because Braxton does not appreciate the problems that occur when attempting to produce and isolate a soluble protein with free cysteines. More particularly, if one attempts to produce a *soluble* cysteine mutant according to the procedure of Braxton, the present inventors have demonstrated that the procedure of Braxton does not predictably result in the desired soluble protein that has free cysteines for further modification. To illustrate Applicants' position, it is noted that Braxton teaches that cysteine muteins of growth hormone can be expressed and "purified according to methods known in the art" for expressing and purifying wild type growth hormone (Example I.2., column 47, lines 1-2.). The preferred method for expressing growth hormone in *E. coli* is to secrete the protein to the periplasmic space, where it can be released from the cell in a soluble, properly folded and biologically active form by osmotic shock treatment of the cells (Koshland and Botstein, Cell 20: 749-760, 1980; Hsiung et al., Biotechnology 4: 991-995, 1986). The present inventors used this method to express and purify wild type growth hormone (Examples 2 and 3 of the present application). However, this method did not prove useful for purifying cysteine muteins of growth hormone (see Example 5 of

the present application). The problem encountered by the present inventors was that the growth hormone cysteine muteins were secreted and soluble (as defined by the fact that the proteins were present in the supernatant fraction following centrifugation of the osmotic shock lysates of the cells), but consisted of multiple molecular weight species (multiple monomeric species and aggregated species). As a result, no clear growth hormone cysteine mutein band could be discerned upon non-reducing SDS-PAGE analysis of the supernatant fraction of the osmotic shock lysate. The multiple molecular weight species and aggregated species could not be purified from the supernatant fraction of the osmotic shock cell lysate using procedures that worked well for wild type growth hormone. Therefore, it is submitted that Braxton also do not teach or suggest any suitable method for the isolation of a soluble protein having at least one free cysteine.

With regard to the presently claimed method, it is noted that, prior to the present inventors' work, it was not obvious that the reason that soluble cysteine mutein proteins were difficult to purify was due to reactivity of the free cysteine residue. Since the proteins were soluble, one of skill in the art would assume that they are properly folded and disulfide bonded. However, as discussed above with regard to growth hormone, production of soluble cysteine muteins using methods that are suitable for wild-type proteins frequently results in the production of multiple molecular weight species (multiple monomeric species and aggregated species). It also was not obvious, prior to the present invention, that adding a cysteine blocking agent (cystine) to the soluble cysteine mutein proteins, in the absence of a denaturing agent and a reducing agent (i.e., the procedure taught by Cox et al.), would result in the free cysteine residue being blocked and aid in protein recovery. It also was not obvious that adding the cysteine blocking agent to a soluble cysteine mutein protein would not disrupt the native disulfide bonds in the protein and reduce the protein's biological activity.

With regard to the particular protein products produced by the claimed method (formerly Claims 20-24), Applicants submit that because none of the cited references teach or suggest the method of the present invention, none of the cited references would be able to teach or suggest any of the claimed proteins, and one would not reasonably predict that the methods cited references could successfully produce the claimed soluble proteins having the recited activity. None of the cited references teach or suggest the recited proteins having the specified biological activities, nor a method by which the recited proteins can be produced. The cited references, alone or in

combination, do not teach or suggest *how* to produce the claimed cysteine muteins having the recited biological activity (i.e., the specific location of the introduced cysteine residue or site of attachment of the PEG molecule) and a method by which such proteins can reliably be produced. Neither of Seeley et al. or Cox et al. teach or suggest the production of the claimed soluble proteins. With regard to Braxton et al., as discussed above, the present inventors were unable to purify any cysteine muteins of growth hormone, erythropoietin or alpha interferon using the methods taught by Braxton. Instead, the present inventors had to develop a novel procedure, as recited in the present claims, to produce the claimed proteins.

In view of the foregoing remarks, Applicants respectfully request that the Examiner withdraw the rejection of Claims 1-25 and 29-31 under 35 U.S.C. § 103.

Applicants have attempted to address all of the issues as set forth in the July 28 Office Action and submit that the claims are in a condition for allowance. In the event that the Examiner has any additional concerns regarding Applicants' position, the Examiner is invited to contact the below-named agent at (303) 863-9700 to expedite the resolution of prosecution.

Respectfully submitted,

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